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Fatty Acids Released by *Chlorella vulgaris* and Their Role in Interference with *Pseudokirchneriella subcapitata*: Experiments and Modelling

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Abstract The role of extracellular fatty acids in the interference between two algae, *Chlorella vulgaris* Beijerink and *Pseudokirchneriella subcapitata* (Korshikov) Hindak, was assessed by the co-cultivation of the two selected strains, as well as by the chemical analysis of exudates from the culture media of single strain cultures. The effect of culture age and phosphate limitation was evaluated. The experiments showed that the composition and amount of fatty acids, released by *C. vulgaris* and by *P. subcapitata*, both in a batch and in a continuous monoculture, depend on the culture age and on the phosphate concentration in the culture medium. We also found that the amount of chlorellin generated in the two algae co-culture increased and was almost exclusively constituted by a mixture of C18 fatty acids. By using the evaluated concentrations of these fatty acids, an artificial chlorellin was prepared. The toxicity of this mixture to *P. subcapitata* appears to be similar to that of the natural chlorellin. For both algae, a stimulation of growth was observed at low concentrations of the natural chlorellin, whereas higher concentrations produced inhibitory effects on both species. However, *P. subcapitata* was much more sensitive than *C. vulgaris*. By using some of these new experimental results, two new mathematical models have been used to describe the toxicity of chlorellin to *C. vulgaris* and to the interference between *C. vulgaris* and *P. subcapitata*, respectively.

Keywords *Chlorella vulgaris* · *Pseudokirchneriella subcapitata* · Chlorellin · Fatty acids · Allelopathy · Mathematical model

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Introduction

The green unicellular coccoid alga *Chlorella vulgaris* Beijerink was the first plant species in which a release of substances affecting other organisms was observed (Pratt and Fong 1940). Further investigations revealed that not a single compound, but a mixture of fatty acids and hydrocarbons (Spoehr et al. 1949), named chlorellin, was responsible for the toxicity towards bacteria and algae (McCracken et al. 1980). Such a joint action seems to be a widespread phenomenon in allelopathy and, according to Einhellig (1996), all allelopathic activities are due to a mixture of two or more compounds. Fatty acid mixtures frequently have been reported toxic in aquatic environments (Ikawa 2004). Chiang et al. (2004) found that blooms of the green unicellular alga *Botryococcus braunii* Kützing produce high amounts of fatty acids, which are severely toxic

to fish. Furthermore, Wu et al. (2006) demonstrated that the cytotoxicity of free fatty acids towards green algae and cyanobacteria affects primarily the plasma membrane.

Generally speaking, it is difficult to assess the role of allelopathy in the interference between two algal populations in the field, because it is difficult to evaluate the bioactive concentration of allelopathic substances, and also because too many environmental factors may influence their release from the donor organism (Blum et al. 1999). Furthermore, the sensitivity of the target species may vary, depending on the mode of action of the allelochemicals and environmental conditions (Inderjit and Duke 2003).

It is therefore more appropriate to study allelopathic phenomena under controlled laboratory conditions, where many variables can be kept constant, provided that the laboratory environment can mimic the natural environment sufficiently. This applies, for instance, for many open aquatic ecosystems, which often can be quite well simulated with chemostat laboratory cultures (Smith and Waltman 1995). This approach has been used previously by Fergola et al. (2007), and is used in the present paper. Fergola et al. (2007) found that *Pseudokirchneriella subcapitata* (Korshikov) Hindak became extinct in mixed cultures with *C. vulgaris* grown under phosphate-limiting conditions. A constant release of chlorellin-like material by *C. vulgaris* was observed during the time course of those experiments, and preliminary analyses confirmed that the exudates were composed primarily of various fatty acids. Here, by employing some new experimental results, two new mathematical models were used to describe the toxicity of chlorellin to *C. vulgaris* and to the interference between *C. vulgaris* and *P. subcapitata*, respectively.

Mathematical modelling is making increasingly significant contributions to many research areas. Mathematical models of biological and biochemical processes often represent a successful tool to better understand complex phenomena. Analysis (mathematical and numerical) can permit a deeper understanding both by predicting new scenarios and by suggesting new experiments. In the context of population dynamics, the influence of toxicants on the growth of populations and on competition among species has been widely studied in the last few years (Hallam et al. 1983; Hallam and Ma 1986, 1987a, b; Freedman et al. 1989; Smith and Waltman 1995; Hsu and Waltman 1998; Mukhopadhyay et al. 1998, 2003; Braselton and Waltman 2001). In particular, importance has been given to the competition that takes place in a chemostat-type environment. Such competitions have been mathematically represented essentially according to two different types of modelling approaches, adopted by Chattopadhyay (Mukhopadhyay et al. 1998, 2003), and Waltman (Smith and Waltman 1995; Braselton and Waltman 2001; Fergola et al. 2004, 2006), respectively. The two models used here follow this latter approach and give a representation of the dynamics of the interference taking place between the two algal species.

The main issue addressed in this study was to assess whether the age of culture, phosphate depletion, or co-occurrence of another species affected the composition of chlorellin and its release by *C. vulgaris*. Moreover, the biological activity of the natural exudate mixtures isolated from the co-culture of *C. vulgaris* and *P. subcapitata*, was compared to artificial chlorellin added to the chemostat culture, obtained by mixing the four main fatty acids in the proportion found in the exudates. On the basis of these results, a data analysis was performed. Additionally, some numerical simulations were performed by assigning to the parameters of the two considered models, numerical values obtained from some of the laboratory experiments described both in this paper and in Fergola et al. (2007).

Methods and Materials

Selection of Strains and Culture Conditions Two axenic strains were used: *C. vulgaris* CCAP 211/11b and *P. subcapitata* UTEX 1648 from ACUF (Algal Collection of the University Federico II, Naples). The algal strains were cultivated for 2 wk on Bold Basal Medium (BBM) (Nichols 1973) with an inorganic phosphate (Pi) concentration of 25 g l^{-1}. For *P*-limited cultures, the Pi concentration was reduced to 0.5 mg l^{-1} (Chen et al. 1997). Cultures were maintained in 100 ml Erlenmeyer flasks, at 23°C on shaking incubators at 40 rpm. Continuous illumination, with an irradiance of 80 μE m^{-2} sec^{-1}, was supplied by cool white fluorescent tubes (Philips TLD 39 w/55). Batch experiments, either with each single species or with mixed cultures of both species, were performed under the same conditions. The growth of population of *C. vulgaris* or *P. subcapitata* was followed using a spectrophotometer (Secoman 250) at λ=550 nm. When the experiments were carried out with mixed culture of the two algae, cells were counted by using a Burker hemocytometer, under an optical microscope (Nikon Eclipse 800).

Chemostat Cultures Algal competition for a limiting nutrient (phosphate) was examined in a chemostat culture system. The culture vessel was a standard chemostat containing 1,200 ml of culture media. Nutrient was pumped at a constant rate into the culture flasks by means of a peristaltic pump (GILSON–MINIPLUS 3) at a rate of 600 ml d^{-1} to give a washout rate of 50% a day. Experiments were carried out either on unialgal or mixed cultures. Continuous illumination (80 μE m^{-2} sec^{-1}) was provided by cool fluorescent lamps (Philips TLD 39 w/55). Sterile humidified air was bubbled through the cultures.

The air flow produced by an air compressor was controlled by a flow-meter and conveyed into two flasks of sterile water. Each culture was stirred continuously with a mixer (Heidolph). All experiments were performed using a BBM medium with a Pi concentration of 0.5 mg l^{-1}, as suggested by Chen et al. (1997). For the competition experiments, the two species were grown initially in different flasks to give a fixed cell number of 300,000 cells ml^{-1} for both strains. Successively, samples of both algae were withdrawn from 4-d-old cultures, and aseptically inoculated in the chemostat.

Analysis of Chlorellin Batch cultures either of *C. vulgaris* or *P. subcapitata* were carried out in 5,000 ml cylinders under continuous light at (80 μE m^{-2} sec^{-1}) at 23°C. The algal cultures were stirred continuously and air bubbled through the cells that were grown under *P*-sufficient (25 g l^{-1}) or *P*-limiting (0.5 mg l^{-1}) conditions. Aliquots of the cultures (2,500 ml) were collected and filtered during the mid-exponential phase (6,650,000 cells ml^{-1}) and at the beginning of the stationary phase of growth (14,000,000 cells ml^{-1}). In the co-cultures grown in the chemostat, the outflow was collected daily and stored at −20°C. All culture media were concentrated with an evaporator to 200 ml, and exhaustively extracted with methylene chloride (3×150 ml; recovery rate ~90%, 8%, <1%, respectively). The organic fraction was concentrated to dryness to obtain a lipoid fraction. This was derivatized with ethereal diazomethane and analyzed with a GC (Gas Chromatography) by using an RTX-5 capillary column (Restek; 30 m×0.25 mm inside diam, flow rate 10 ml min^{-1}, N_2 used as carrier gas). Analysis was performed with the following temperature program: 140°C for 5 min, from 140°C to 240°C at 4°C min^{-1}, and 240°C for 60 min. Fatty acid methyl esters (FAMEs) were identified by comparing their retention times with those of 19 commercial fatty acid standards purchased from SUPELCO, with the Limit of Quantitation of 14 ppb. Fatty acids yield and composition were studied also in co-cultures of *C. vulgaris* and *P. subcapitata* grown in *P*-limiting cultures. Individual algae from continuous cultures (300,000 cells ml^{-1}) were mixed at a 1:1 ratio, and after 4 d, when *C. vulgaris* was at a concentration of 1,200,000 cells ml^{-1} and *P. subcapitata* at 100,000 cells ml^{-1}, the composition of the medium was analyzed.

Toxicity Bioassays In order to analyze the composition and evaluate the toxicity of natural chlorellin, the culture medium (4 l) of *C. vulgaris* and *P. subcapitata* co-cultures was collected, concentrated with an evaporator to 200 ml, and exhaustively extracted with methylene chloride (3× 150 ml). The mixture of fatty acids extracted was dried and re-dissolved in 5 ml of dimethyl sulfoxide (DMSO). The solution was stirred for 2 h in the dark at room temperature. Test solutions were prepared by mixing the appropriate volumes of the DMSO stock solutions with culture media. The concentration of DMSO in culture media was 2 ml l^{-1} at the highest concentration of chlorellin tested (52 mg l^{-1}). Liquid growth inhibition tests against this extracted, natural mixture of fatty acids were performed with *P. subcapitata* or *C. vulgaris*. Inocula corresponding to 10,000 cells ml^{-1} (ISO protocol 1987) from cultures of each strain in mid exponential phase (4-d-old) were grown in 100 ml Erlenmeyer flasks containing 50 ml of BBM. Liquid inhibition tests also were carried out with chemostat cultures of *P. subcapitata* with the same initial cell concentration (300,000 cells ml^{-1}) against chlorellin at a constant concentration of 1.90 mg l^{-1}, and number of cells, photosynthetic rate, and Chlorophyll *a* concentration were monitored daily. For this purpose, pre-cultures of *P. subcapitata* were grown on *P*-sufficient BBM medium (concentration mg l^{-1}). When the algae were in mid-exponential growth phase (4-d-old), an inoculum (10 ml) was centrifuged, washed two times in sterile 0.9% NaCl solution, and then added to a culture vessel of a standard chemostat containing 1,200 ml of *P*-limited BBM (Pi 0.5 mg l^{-1}). Algae were grown in the chemostat according to the conditions described above (section *Chemostat Cultures*). Preliminary tests carried out on *P. subcapitata* gave evidence that cells that have been treated according to this protocol and re-suspended in *P*-sufficient BBM grew at rates comparable to those of control cultures. To assess the relationship between fatty acid toxicity and cell concentrations at very low cell densities, *P. subcapitata* cultures at an initial inoculum concentration ranging from 500 to 5,000 cells ml^{-1} were exposed to 1.90 mg l^{-1} of chlorellin isolated from co-cultures of *C. vulgaris* and *P. subcapitata*.

Fatty acids extracted from the medium of co-cultures of *C. vulgaris* and *P. subcapitata* were tested at concentrations ranging from 0.40 to 52 mg l^{-1}. A mixture of the four main fatty acids found in the natural chlorellin extract also was prepared by using stearic acid (C18:0, >99%), oleic acid (C18:1n9c, >98%), linoleic acid (C18:2n6c, 97%), and linolenic acid (C18:3n3c, 99%) (Sigma–Aldrich, Germany). It was dissolved in DMSO to give the same ratio found in a natural extract (linoleic acid 46.4%, linolenic acid 21.0%, oleic acid 19.2%, and stearic acid 13.2%). All bioassays and BBM analyses, controls and positive controls (containing only 2 ml l^{-1} DMSO) were carried out in triplicate in axenic conditions at 23°C with continuous illumination of 80 μE m^{-2} sec^{-1}. At the end of the incubation, the final pH was also measured. No difference in growth was observed among controls. Chlorophyll *a* was measured according to Lazzara et al. (1990), and photosynthetic rate was measured according to Pollio et al. (1993).

Statistical Evaluation For liquid growth inhibition tests, the effect of concentration, which differs significantly from that of the control, was determined by multi comparison Dunnett's tests, after verifying the Shapiro–Wilk's test for normality and the Hartley's test for homogeneity of variance ($P>0.5$). Calculations were performed using TOXSTAT 3.0 software (Gulley et al. 1989).

Mathematical Modelling Here, we introduce two deterministic mathematical models constituted by Ordinary Differential Equations, which represent the influence of chlorellin on *C. vulgaris* and *P. subcapitata* both in mono- and mixed culture. The two models are based on data obtained with several experiments performed in our laboratories. Our experiments gave us the opportunity to understand and quantitatively represent the following four crucial features (Fergola et al. 2007): a) nutrient uptake rates; b) yields (ratios between the amounts of produced biomass and absorbed nutrient); c) rate of allelochemical production; and d) the inhibitory effects of allelochemicals.

Mathematical Modelling of Inhibition Effects of Chlorellin We tested two mathematical models, both based on experimental data, to represent the inhibition effects of chlorellin on *P. subcapitata* and *C. vulgaris*. For the inhibitory effects on the growth rate of *P. subcapitata*, Eq. (1) was a suitable model fitting the experimental data (Fergola et al. 2007):

$$f_1(S)e^{-\gamma p} \tag{1}$$

where $f_1(S)$ is the growth function in the absence of toxicant, p is the concentration of chlorellin in the environment, and γ is a measure of the inhibiting effect of chlorellin (γ=7.81 l^2 mg^{-2}). In Fergola et al. (2007) the influence of chlorellin on *C. vulgaris* was not included. Here, on the basis of new experiments, this process is taken into account and Eq. (2) is proposed as a suitable representation of the growth rate of *C. vulgaris* (LAB Fit software):

$$f_2(S)e^{-\alpha p^2} \tag{2}$$

where $f_2(S)$ is the growth function in absence of toxicant and computed at a given value of the nutrient concentration, and α is a measure of the inhibiting effect of chlorellin $\alpha>0$).

The estimate of the parameter α is chosen to minimize the χ^2 merit function given by the sum of squared residuals $\sum_i e_i^2$. This iterative fitting procedure is based on a modified Levenberg–Marquardt algorithm (Meyer and Roth 1972).

The Effect of Chlorellin on C. vulgaris in Chemostat Culture In order to describe the growth of *C. vulgaris* we considered three Ordinary Differential Equations (ODE), which represent the mass balance equations for the concentrations respectively of:

the nutrient,

$$\frac{dS}{dt} = \left(S^0 - S\right)D - f(S)e^{-\alpha p^2}\frac{N}{\eta} \tag{3}$$

the algae,

$$\frac{dN}{dt} = N\left[(1-kN)f(S)e^{-\alpha p^2} - D\right] \tag{4}$$

and the chlorellin,

$$\frac{dp}{dt} = kf(S)e^{-\alpha p^2}N^2 - Dp \tag{5}$$

where d/dt denotes the time derivative, $S = S(t)$ is the phosphate concentration at time t, $N = N(t)$ is the biomass of *C. vulgaris* at time t, $p = p(t)$ is the concentration of chlorellin in the environment at time t, η is the constant yield of the population (Smith and Waltman 1995), S^0 is the constant input rate of the limiting nutrient concentration ($S^0>0$), D is the constant washout rate ($D>0$), $f(S) = \frac{mS}{a+S}$ is the functional response of *C. vulgaris* (Michaelis–Menten model) with the semi-saturation constant a ($a>0$), and m ($m>0$) the maximal specific growth rate of *C. vulgaris*, and α is a measure of the inhibiting effect of chlorellin ($\alpha>0$).

Finally we note that, according to Fergola et al. (2007), the term $kNf(S)$ is the rate of allelochemical production and is represented by the fraction of potential growth devoted to produce the chlorellin ($0<kN<1$). As in Fergola et al. (2007) we assume that the overall energy available to *C. vulgaris* arises only from nutrient consumption. Moreover, it is not completely available for the algae growing process, but is devoted partially to the production of chlorellin. In other words, we assume that a sort of energy conservation law holds, and furthermore, that the production of allelochemicals is proportional to the concentration of algae and thus represented by a linear function, which includes the constant k.

By performing the following scaling of the parameters (Smith and Waltman 1995)

$$\begin{aligned} &S = \overline{S}S^0, N = \overline{N}\eta S^0, p = \overline{p}S^0\eta, m = \overline{m}D, t = \frac{\tau}{D}, \\ &a = \overline{a}S^0, \alpha = \frac{\overline{\alpha}}{S^{02}\eta^2}, f(S) = \overline{f}\left(\overline{S}S^0\right), \overline{k} = k\eta S^0 \end{aligned} \tag{6}$$

we obtain from the Eqs. (3)–(5) a dimensionless system for the state variables S, N, p, which can be written, by removing the bars, in the form

$$\begin{cases} \frac{dS}{dt} = 1 - S - f(S)e^{-\alpha p^2}N \\ \frac{dN}{dt} = N\left[(1-kN)f(S)e^{-\alpha p^2} - 1\right] \\ \frac{dp}{dt} = kf(S)e^{-\alpha p^2}N^2 - p \end{cases} \tag{7}$$

By standard techniques, we can prove the following two lemmas:

Lemma 1 Any solution of (7), with positive initial conditions, remains positive whenever it exists.

Lemma 2 Any solution of (7) is bounded (See proof in Appendix).

In view of the results concerning the growth of species in a chemostat-like environment (Smith and Waltman 1995), we suppose $m_i>1$, $i=1, 2$ and state the following theorem without the proof.

Theorem 1 System (7) admits the steady state solution E=(1, 0, 0) for all values of the parameters.

Theorem 2 If $\frac{m}{a+1} > 1$ then system (7) admits one positive equilibrium $E^*=(S^*, N^*, p^*)$. (See proof in Appendix)

Local Stability The stability analysis of the generic equilibrium $\overline{E} = (\overline{S}, \overline{N}, \overline{p})$ can be performed by means of the characteristic equation associated to the linearized system of (7)

$$\begin{cases} \dot{x}_1 = \left(-1 - f'(\overline{S})\overline{N}e^{-\alpha\overline{p}^2}\right)x_1 - f(\overline{S})e^{-\alpha\overline{p}^2}x_2 + 2\alpha f(\overline{S})\overline{p}\overline{N}e^{-\alpha\overline{p}^2}x_3 \\ \dot{x}_2 = f'(\overline{S})\overline{N}e^{-\alpha\overline{p}^2}(1-k\overline{N})x_1 + \left[(1-2k\overline{N})f(\overline{S})e^{-\alpha\overline{p}^2} - 1\right]x_2 - 2\alpha\overline{p}\overline{N}(1-k\overline{N})f(\overline{S})e^{-\alpha\overline{p}^2}x_3 \\ \dot{x}_3 = f'(\overline{S})\overline{N}^2 k e^{-\alpha\overline{p}^2}x_1 + 2k\overline{N}f(\overline{S})e^{-\alpha\overline{p}^2}x_2 + \left(-2\alpha\overline{p}\overline{N}kf(\overline{S})e^{-\alpha\overline{p}^2} - 1\right)x_3 \end{cases} \tag{8}$$

whose characteristic equation can be written as follows

$$\det\begin{bmatrix} \left(-1 - f'(\overline{S})\overline{N}e^{-\alpha\overline{p}^2}\right) - \rho & -f(\overline{S})e^{-\alpha\overline{p}^2} & 2\alpha f(\overline{S})p\overline{N}e^{-\alpha\overline{p}^2} \\ f'(\overline{S})\overline{N}e^{-\alpha\overline{p}^2}(1-k\overline{N}) & \left[(1-2k\overline{N})f(\overline{S})e^{-\alpha\overline{p}^2} - 1\right] - \rho & -2\alpha\overline{p}\overline{N}(1-k\overline{N})f(\overline{S})e^{-\alpha\overline{p}^2} \\ f'(\overline{S})\overline{N}^2 k e^{-\alpha\overline{p}^2} & 2k\overline{N}f(\overline{S})e^{-\alpha\overline{p}^2} & -2\alpha\overline{p}\overline{N}kf(\overline{S})e^{-\alpha\overline{p}^2} - 1 - \rho \end{bmatrix} = 0. \tag{9}$$

We can prove that:

Theorem 3 The following statements hold true: (See proof in Appendix)

(i) If $m < a + 1$, then the equilibrium E_0 turns out locally asymptotically stable;
(ii) If E^* exists, then it turns out locally asymptotically stable.

A New Model for the Interference between C. vulgaris and P. subcapitata In the system (3) of Fergola et al. (2007) the influence of chlorellin on *C. vulgaris* was not included. Here, according to the previous section, we assume that chlorellin also can act on *C. vulgaris*, and we propose to model the interference between the two algae with the following new system

$$\begin{cases} \frac{dS}{dt} = (S^0 - S)D - f_1(S)e^{-\gamma p}\frac{N_1}{\eta_1} - f_2(S)e^{-\alpha p 2}\frac{N_2}{\eta_2} \\ \frac{dN_1}{dt} = N_1[f_1(S)e^{-\gamma p} - D] \\ \frac{dN_2}{dt} = N_2\left[(1-kN_2)f_2(S)e^{-\alpha p 2} - D\right] \\ \frac{dp}{dt} = kf_2(S)e^{-\alpha p 2}N_2^2 - Dp \end{cases} \tag{10}$$

where the inhibition effects of chlorellin on the two algae have been modeled in different ways and, according to what was suggested in the Discussion in Fergola et al. (2007), the two functions $f_1(S)$ and $f_2(S)$ are both of the Michaelis–Menten type.

Results

Concentration and Composition of Fatty Acids Released by C. vulgaris under Different Culture Conditions Experiments evaluated whether the chlorellin production of *C. vulgaris* is affected by age of culture, phosphate concentration in the culture medium, or co-occurrence of another algal strain. The lipoidic material recovered from the medium (*P*-sufficient) of *C. vulgaris* was 0.41 mg l^{-1} 10^{-6} cells in mid-exponential phase cultures and 0.70 mg l^{-1} 10^{-6} cells in stationary phase cultures. From *P. subcapitata* cultures, 0.08 mg l^{-1} 10^{-6} cells in the mid-exponential phase and 0.11 mg l^{-1} 10^{-6} cells in the stationary phase of growth were recovered (Table 1).

The GC analysis of the exudates showed the presence of fatty acids, identified by comparison with commercial standards. Under *P*-sufficient conditions, *C. vulgaris* batch cultures released fatty acids, ranging from C8 to C18, during their exponential growth phase, but a significantly

Table 1 Amounts of fatty acids (mg/l/10^6 cells) recovered from culture medium of *Chlorella vulgaris* Beijerink and *Psuedokirchneriella subcapitata* (Korshikov) Hindak. a) exponential growth phase, batch culture, *P*-sufficient; b) stationary growth phase, batch culture, *P*-sufficient; c) exponential growth phase, chemostat culture, *P*-limiting conditions; d) a mixed culture of *C. vulgaris* and *P. subcapitata* (initial ratio 1:1) in chemostat under *P*-limiting conditions, exponential growth phase

C. vulgaris			*P. subcapitata*			*C. vulgaris P. subcapitata*
a	b	c	a	b	c	d
0.41	0.70	0.85	0.08	0.11	0.25	1.90

changed fatty acid composition during their stationary phase that showed a higher degree of polymerization (C14 to C22 acids). *Pseudokirchneriella subcapitata* cultures produced, during the mid-exponential growth phase, a mixture whose major components were C12, C18, C20, and C22 fatty acids; the same compounds, except C20 and C22, also were found in the medium collected from the stationary phase of growth.

These data indicate that the amounts and kinds of fatty acids were different for the two algae cultures (Tables 1, 2). The experiments on a single culture of the selected algae also were carried out in a chemostat, under *P*-limiting conditions. Fatty acids isolated from *C. vulgaris* under these conditions yielded 0.85 mg l^{-1} 10^{-6} cells (Table 1). Their composition was compared with that found in *P*-sufficient cultures during the exponential and stationary growth phases. The comparison showed an increase of acid compounds ranging from C8 to C18 (Table 2). Additionally, in the *P. subcapitata* culture, an increase of fatty acid concentrations was observed (0.25 mg l^{-1} 10^{-6} cells) (Table 1) in respect to the values obtained from batch cultures, and the fatty acid composition was represented by different compounds ranging from C8 to C20 (Table 2).

Fatty acid yield and composition also were studied in co-cultures of *C. vulgaris* and *P. subcapitata* grown in *P*-limiting cultures and with an initial inoculum of 300,000 cell ml^{-1} each. After four days, the composition of the medium was analyzed. At that time, we observed an increased growth of *C. vulgaris* (1,200,000 cells ml^{-1}) and a partial extinction of *P. subcapitata* (100,000 cells ml^{-1}). The isolated fatty acid mixture yielded 1.90 mg l^{-1} 10^{-6} cells ml^{-1}, which was the highest fatty acid concentration observed in our experiments (Table 1). The co-culture of the two algae grown under phosphorus deficiency also influenced the fatty acid composition of the mixture found in the culture medium (Table 2), which in this case

Table 2 Qualitative composition of the fatty acid mixture recovered from different culture media and growth conditions of *Chlorella vulgaris* and *Pseudokirchneriella subcapitata*. a) exponential growth phase, batch culture, *P* sufficient; b) stationary growth phase, batch culture, *P* sufficient; c) exponential growth phase, chemostat culture, *P*-limiting conditions; d) a mixed culture of *C. vulgaris* and *P. subcapitata* (initial ratio 1:1) in chemostat under *P*-limiting conditions; (x = present; ± = in trace)

Fatty acids	*C. vulgaris*			*P. subcapitata*			*C. vulgaris P. subcapitata* (ratio 1:1)
	a	b	c	a	b	c	d
C8:0	x		x			x	
C10:0			x				
C12:0			x	x	x	x	
C13:0	x		x				
C14:0		x				x	
C14:1n9c	x		x	±	±		
C15:0			x	±	±	x	
C16:0				±	±	x	
C16:1n9c		x	x		±		
C17:0	x	x	x	±		x	x
C18:0		x	x	±	±		x
C18:1n9c		x	x	±	x		x
C18:1n9t		x	x	±	x		x
C18:2n6c		x	x	x	x	x	x
C18:3n3c		x	x				x
C20:0		x					x
C20:1n9c		x	x	x		x	
C22:0			x			x	
C22:1n9c		x	x	x			

x=>14 ppb, ±=<14 ppb

was constituted almost exclusively by the following C18 fatty acids: stearic acid (13.2%), oleic acid (19.2%), linoleic acid (46.4%), and linolenic acid (21.0%).

Effect of Fatty Acids Mixture on C. vulgaris and P. subcapitata Growth The toxicity of chlorellin isolated from the co-culture of the two algae was assayed separately either on *P. subcapitata* or *C. vulgaris* grown in batch cultures. The range of concentrations tested was 0.4–52 mg l^{-1}. Figure 1 shows the concentration-response relationship of the two algae following 96 h exposure to the fatty acid mixtures. A stimulation of growth at low concentrations of chlorellin was observed for both algae. However, *P. subcapitata* was much more sensitive than *C. vulgaris* to chlorellin concentration. In the case of this latter organism, fatty acids elicited slight growth stimulation at concentrations ranging from 0.4 to 13.0 mg l^{-1}, and exerted a toxic action only at the highest concentrations tested, whereas *P. subcapitata* growth was appreciably stimulated only at the lowest concentrations tested and was strongly inhibited from 6.5 mg l^{-1} onward.

Although chlorellin isolated from algal co-cultures contained traces of other unidentified substances, we prepared a synthetic mixture of the four commercially available C18 fatty acids only, at the same ratio found in natural exudates. The mixture was assayed against *P. subcapitata* and *C. vulgaris*, giving the results reported in Fig. 2. For both species, we observed a growth stimulation at low concentrations and an inhibitory effects at high concentration. Kruskal–Wallis one way analysis of variance on ranks showed that there was no significant difference between the toxicity of chlorellin and the artificial fatty acid mixtures (P=0.171). The results shown in Figs. 1 and 2 also indicate that, under the experimental conditions of batch assays, a concentration of 1.90 mg l^{-1} of the fatty acid mixture was not toxic to *P. subcapitata* (1.90 mg l^{-1} is the amount found in co-culture). Two further experiments were carried out to better understand whether or not even lower cell densities are affected by such a concentration of fatty acids. In the first, the relationship between fatty acid

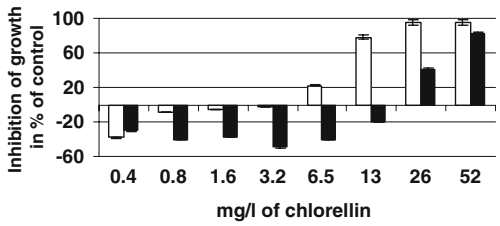

Fig. 1 Effects of chlorellin isolated from co-cultures of the two algae on *Chlorella vulgaris* or *Psuedokirchneriella subcapitata* growth (after 96 h exposure), grown in chemostat under *P*-limiting conditions. (black square *C. vulgaris*; white square *P. subcapitata*); *error bars* indicate the standard deviation

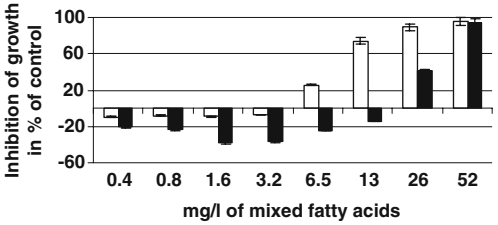

Fig. 2 Effects of fatty acids mixture (stearic, oleic, linoleic and linolenic acids) on *Chlorella vulgaris* or *Pseudokirchneriella subcapitata* growth (after 96 h exposure), grown in chemostat under *P*-limiting conditions; (black square *C. vulgaris*; white square *P. subcapitata*); *error bars* indicate the standard deviation

toxicity and cell concentration showed no inhibition of growth in any of the inocula tested. In the second, the influence of *P*-limiting conditions together with a chlorellin concentration of 1.90 mg l^{-1} was evaluated on *P. subcapitata* grown in a chemostat. Although the experimental environment was equivalent to that described for previous experiments of co-culture with *C. vulgaris*, no inhibition was observed. The results of the experiments were used to develop a revised mathematical model that describes the nature of interference between *C. vulgaris* and *P. subcapitata*.

Mathematical Modelling The model (2) was fitted to the experimental data contained in Table 3 by using a non-linear minimization function (Nonlinear Regress) of the software package Mathematica (Wolfram-Research 1988). In this way, we computed α=4.38 l^2 mg^{-2} with an asymptotic standard error of 0.75 (Fig. 3). The variance was estimated to be 0.2×10^{-2}.

With respect to other models quoted in literature, the two considered here [(1), (2)] were selected mainly because of the advantage of mathematical tractability, simplicity, and the lowest residual standard error. The numerical simulation obtained from Eqs. (3)–(5) is given in Fig. 4. It shows good agreement between the experimental data of *C. vulgaris* growth in the chemostat and the numerical curve.

In a previous study (Fergola et al. 2007), we performed simulations of co-cultures of *C. vulgaris* and *P. subcapitata*, grown in a chemostat under phosphorus limitation. We found that *P. subcapitata* was excluded from the culture within nine days. The model developed to describe the interference between the two algae suggested that both competition for nutrients and toxic action of fatty acids, excreted by *C. vulgaris*, were responsible for the final exclusion of *P. subcapitata*.

For this investigation, some numerical simulations were performed. However, by comparing the numerical simulations obtained through system (10) of this paper with the experimental data of the laboratory interference, currently collected, there was not much difference from that obtained using system (3) of the Fergola et al. (2007).

Table 3 The experimental *Chlorella vulgaris* growth rates in correspondence of different toxicant concentrations

Chlorellin (mg/l 10^2)	0	0.002	0.004	0.0078	0.0156	0.03125	0.0625	0.125	0.25	0.52
Growth rate (d^{-1})	0.64	0.70	0.67	0.70	0.71	0.72	0.72	0.69	0.41	0.19

In other words, the effect of chlorellin on *C. vulgaris* in system (10) does not seem to play an important role on the interference between the two algae, unless, once again, the amount of chlorellin produced throughout the experiment was so small that it was not sufficient to produce an observable inhibitory effect. It should be noted, however, that in contrast to the outcome of the laboratory experiments in mono-culture with synthetic chlorellin, the mathematical model predicts, in the experiment in co-culture, an inhibition of the *P. subcapitata* growth by the naturally produced chlorellin.

Discussion

One of the points addressed in this study deals with the chemical characterization of chlorellin. Since the works of Spoehr et al. (1949) and Scutt (1964), only few contributions to the characterization of this mixture have been attempted (Sushchik et al. 2001). Moreover, little information is available on environmental factors that influence the production and release of chlorellin. Sushchik et al. (2003) reported that the composition of extracellular free fatty acids released by *C. vulgaris* did not vary when the algae were grown at different temperatures. The results of our study suggest that the production and the composition of released fatty acids by *C. vulgaris* and *P. subcapitata* are influenced by *P*-supply and/or growth phases, and also by the presence and absence of a competitor.

The production of fatty acids by *C. vulgaris* was increased under P limitation, as also observed in the case of the extracellular release of allelochemicals in the cyanobacterium *Trichormus dolium* (von Elert and Jüttner 1997). In this respect, the observed rise in the production of fatty acids during the stationary phase of growth of the alga could not be only a consequence of cellular senescence but also of the reduced concentration of phosphate in the medium. Another question highlighted by our experiments is the role of the target species on fatty acids released by *C. vulgaris*. The highest fatty acids concentration was found when *C. vulgaris* was co-cultured with *P. subcapitata* under P limitation. The tests carried out on individual cultures of *P. subcapitata* grown under different conditions have shown that it released negligible amounts of fatty acids in the medium, irrespective of phosphate availability and age of the cultures. On the other hand, we cannot rule out the possibility that biocommunicators (Macias 1995; Macias et al. 2008) could be secreted by *C. vulgaris*, thus causing an enhanced release of fatty acids by *P. subcapitata*.

The profile of the fatty acid mixture was shown to be dependent on cultural conditions. Our data indicate that there was a shift towards C18 fatty acids when *C. vulgaris* was cultured under stress conditions. Furthermore the fatty acids mixture isolated from *C. vulgaris* culture under *P*-depleting conditions and in the presence of the competitor *P. subcapitata* was composed mainly of four C18 fatty acids with different degrees of unsaturation. These compounds have been found to be major components of the toxic mixtures released by a number of microalgae

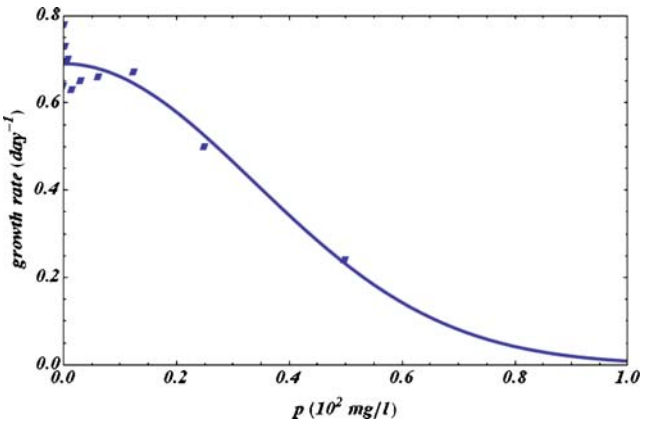

Fig. 3 Dependence of *Chlorella vulgaris* growth rate on the chlorellin concentration p. Comparison between experimental data and the exponential function $f_2(S)e^{-\alpha p^2}$ where α=4.38 (l^2/mg^2)

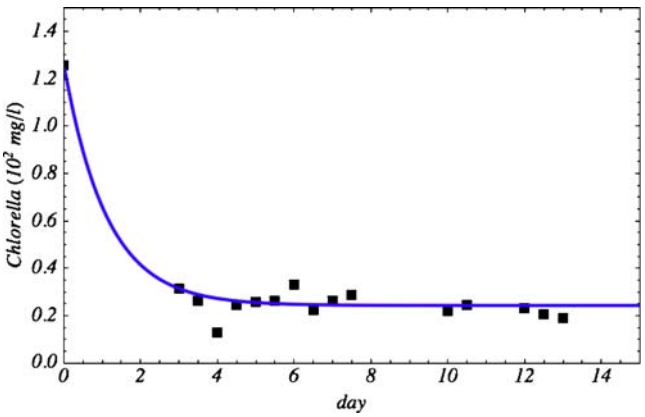

Fig. 4 In the picture, the *points* represent the chemostat experimental data and the *continuous line* is obtained through numerical simulations of Eqs. (3)–(5) with the following parameter values: D=0.9, S^0=0.52, m=1.16, a=0.004, k=0.4, η=0.5, α=4.38

belonging to Chlorophyta, such as *Chlamydomonas reinhardtii* (McCracken et al. 1980), *Chlorococcus* sp., and *Dunaliella primolecta* (Ohta et al. 1995).

The toxicity of fatty acids often has been attributed to their degradation products derived through photoxidation (Spoehr et al. 1949; Murata et al. 1989), but Aliotta et al. (1990) showed that an inhibitory activity of free fatty acids cannot be ruled out. Recently, Bosma et al. (2008) reported that the growth of the Xanthophyte *Monodus subterraneus* was inhibited severely by palmitoleic and oleic acids.

There is increasing evidence of the ecological role played by allelopathic interference in aquatic ecosystems and particularly by fatty acids and their products of photoxidation (Gross 2003, Ianora et al. 2006). However, their effective concentration under natural conditions remains of pivotal importance. The concentration of the fatty acid mixture isolated from single species culture of *C. vulgaris* varied from 0.4 to 0.85 mg l^{-1} in our experiments. In the mixed culture (*C. vulgaris* and *P. subcapitata*), the concentration was much higher (1.90 mg l^{-1}), which is close to those found in some freshwater lakes, where fatty acids are found within a range of 1 and 2 mg l^{-1} (Ikawa 2004). Concentrations of about 0.5 mg l^{-1} were inhibitory to *Phormidium tenue* (Yamada et al. 1993), but it is known that different species exhibit different sensitivities to fatty acid mixtures (Figueredo et al. 2007). In our experiments, chlorellin at 1.90 mg l^{-1} did not significantly influence the growth of *P. subcapitata*. However, in the field, a long exposure to sub-lethal concentrations of fatty acids could affect microalgal species succession. As demonstrated for other classes of compounds (Ianora et al. 2006), there is a high species-specific variability in the effects of allelochemicals on microalgae. Low concentrations of a metabolite can stimulate the growth-rate of some species, but also can be slowly accumulated within the cell, leading to adverse effects in other less resistant species (Ikawa 2004).

The experiments have shown that in the presence of chlorellin the growth of the two algae is modified. To be more precise, we observed that it is slightly stimulated at low concentrations and strongly inhibited as the chlorellin concentration increases. In the models, we did not include the stimulating effect of chlorellin on the two algae, since related experiments are currently in progress.

The simulations obtained on the basis of the suggested mathematical models predicted the experimental results well. Therefore, we reasonably conclude the following:

1) Chlorellin at concentrations above 6.5 mg l^{-1} is autotoxic for *C. vulgaris*. The autotoxic action of a free fatty acid mixture also should be evaluated in the field because, for diatom-derived unsaturated aldehydes, it has been demonstrated that they can act as a diffusible bloom-termination signal (Ianora et al. 2006).
2) *Pseudokirchneriella subcapitata* does not produce sufficient amounts of chlorellin in single species cultures to be ineffective in the competition of the two algal species under chemostat-like conditions. However, experiments are necessary to exclude the idea that biocommunicators produced by *C. vulgaris* or other parameters could enhance the productivity of chlorellin by *P. subcapitata* in co-cultures.
3) The inhibitory effects of a given concentration of natural chlorellin on *P. subcapitata* are similar and qualitatively equivalent to those produced by the same concentration of synthetic chlorellin, prepared as a mixture of the four C18 fatty acids (stearic, oleic, linoleic, and linolenic acids). This is relevant for future experiments, because it allows the use of artificial chlorellin, which is available in large amounts, instead of natural chlorellin, which is usually produced in very small quantities (Fergola et al. 2007).

Appendix

Proof (Lemma 2) If we set

$$z(t) = S(t) + N(t) + p(t) \tag{11}$$

due to system (7), we get

$$\dot{z}(t) = 1 - S - N - p = 1 - z(t), \tag{12}$$

and by integrating from 0 to t, we obtain

$$z(t) = z(0)e^{-t} + 1 - e^{-t},$$

and then

$$\lim_{t \to +\infty} z(t) = 1 \tag{13}$$

thus proving, for any initial condition, the boundedness of the solutions of (7).

Proof(Theorem 2) In order to prove the theorem, we look for positive steady state solutions ($S^*>0$, $N^*>0$, $p^*>0$) and we solve the system obtained by setting the right-hand side of system (7) equal to zero. In this way, we get

$$N^* = \frac{1 - S^*}{k\left(1 - S^*\right) + 1}, \qquad p^* = \frac{k\left(1 - S^*\right)^2}{k\left(1 - S^*\right) + 1} \tag{14}$$

and

$$\left(1 - S^*\right)\left[1 - \frac{f\left(S^*\right)}{k\left(1 - S^*\right) + 1} e^{-\alpha\left(\frac{k\left(1-S^*\right)^2}{k\left(1-S^*\right)+1}\right)^2}\right] = 0 \tag{15}$$

with a few calculations from (14) we find that $N^*>0$, $p^*>0$, if and only if $S^*<1$. We have to exclude the value $S^*=1$

because it makes both p^* and N^* equal to zero. Therefore (by omitting the star), we study the solutions of the equation

$$k(1-S)+1-f(S)e^{-\alpha\left(\frac{k(1-S)^2}{k(1-S)+1}\right)^2}=0 \tag{16}$$

if we define

$$h(S)=k(1-S)+1 \quad \text{and} \quad g(S)=f(S)e^{-\alpha\left(\frac{k(1-S)^2}{k(1-S)+1}\right)^2} \tag{17}$$

then the problem of finding the solution of (15) is changed into finding the solution of $h(S)=g(S)$. Remembering that $S<1$, it is easy to show that in the interval [0, 1], $h(S)$ is strictly decreasing whereas $g(S)$ is strictly increasing.

Therefore, because $h(1)=1$ and $g(1)=\frac{m}{a+1}$ the two curves admit only one intersection point with abscissa $S^*<1$ (Fig. 5).

Proof (Theorem 3)

(i) The roots of Eq. (9) computed in $E_0=(1, 0, 0)$ are

$$\rho_1=-1,\ \ \rho_2=f(1)-1,\ \ \rho_3=-1.$$

Therefore, the steady state E_0 turns out asymptotically stable if

$$\rho_2<0\Rightarrow f(1)<1 \text{ that is } m<a+1.$$

(ii) In $E^*=(S^*, N^*, p^*)$, Eq. (9) can be written as follows

$$\begin{aligned}&\rho^3+(a_{11}+a_{22}+a_{33})\rho^2\\&+(a_{12}a_{21}+a_{11}a_{22}-a_{13}a_{31}+a_{23}a_{32}+a_{11}a_{33}+a_{22}a_{33})\rho+\\&+a_{12}a_{21}a_{33}+a_{11}a_{22}a_{33}-a_{12}a_{23}a_{31}+a_{11}a_{23}a_{32}\\&-a_{13}a_{22}a_{31}-a_{13}a_{21}a_{32}=0\end{aligned} \tag{18}$$

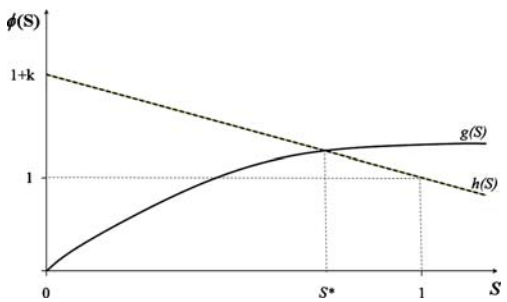

Fig. 5 Here we represent the plots of the two functions $h(S)$ and $g(S)$ defined in (12) in the proof of Theorem 4. These *curves*, as shown, admit only one intersection point with abscissa $S^*<1$

where

$$a_{11}=-\frac{a+S^{*2}}{S^*(a+S^*)},\ a_{12}=-\frac{1-S^*}{N^*},\ a_{13}=2\alpha p^*\left(1-S^*\right),$$
$$a_{21}=\frac{aN^*}{S^*(a+S^*)},\ a_{22}=-k\left(1-S^*\right),\ a_{23}=-2\alpha p^*N^*,$$
$$a_{31}=\frac{ap^*}{S^*(a+S^*)},\ a_{32}=2k\left(1-S^*\right),\ a_{33}=-1-2\alpha p^{*2}$$

are positive constants. In order to study the stability properties of the equilibrium, we can use the Routh–Hurwitz criterion. We observe that, being

1. $a_{11}+a_{22}+a_{33}>0$
2. $$\begin{aligned}&a_{12}a_{21}a_{33}+a_{11}a_{22}a_{33}-a_{12}a_{23}a_{31}+a_{11}a_{23}a_{32}-a_{13}a_{22}a_{31}\\&-a_{13}a_{21}a_{32}\\&=\frac{-\left(1-S^*\right)\left(-\frac{a+ak+kS^{*2}}{S^*(a+S^*)}-2kp^*\left(p^*+2N^*\right)\right)}{\alpha}>0\end{aligned}$$
3. $$\begin{aligned}&(a_{11}+a_{22}+a_{33})\\&(a_{12}a_{21}+a_{11}a_{22}-a_{13}a_{31}+a_{23}a_{32}+a_{11}a_{33}+a_{22}a_{33})\\&-(a_{12}a_{21}a_{33}+a_{11}a_{22}a_{33}-a_{12}a_{23}a_{31}+a_{11}a_{23}a_{32}+-a_{13}a_{22}a_{31}\\&-a_{13}a_{21}a_{32})>0\end{aligned}$$

then all the hypotheses of the Routh–Hurwitz criterion are satisfied and the local stability of the equilibrium E^* follows.

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